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Protein sequence MOTIFS

Protein kinase recognition sequence motifs

Bruce E. Kemp and Richard B. Pearson

Protein kinases play a crucial role in the regulation of many cellular processes. They alter the functions of their target proteins by phosphorylating specific serine, threonine and tyrosine residues. Identification of phosphorylation site sequences and studies with corresponding model peptides have provided clues to how these important enzymes recognize their substrate proteins. This knowledge has made it possible to identify potential sites of phosphorylation in newly sequenced proteins as well as to construct specific model substrates and inhibitors.

A REVIEW of protein kinase recognition sequences is both long overdue and premature. It is overdue because many of the approaches and applications of this field are more than a decade old, and it is premature because we do not know the three-dimensional structure of a single protein kinase substrate complex. The study of protein kinases over the last 35 years has resulted in protein phosphorylation being recognized as one of the most important mechanisms of regulating intracellular processes. There are few, if any, physiological processes in eukaryotes that are not dependent on protein phosphorylation. While this brief review is focused on protein kinase recognition motifs, it should be recognized that protein phosphatases, which catalyse the reverse reaction, are equally important players in the overall process of regulation of protein function by phosphorylation. Undoubtedly, their specificity and regulatory properties are no less important.

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All protein kinases contain a common catalytic domain which typically extends over 240 residues[1], including the binding sites for ATP and the protein substrate (Fig. 1). The ATP-binding site is located at the amino terminus of the domain as characterized by the Rossmann motif, GXGXXG, while the centrally located Asp184 is responsible for base catalysed transfer of the phosphate to the protein substrate[2]. The binding site for the protein substrate is not unequivocally established, although some evidence favours the idea that it is located in the carboxy-terminal 60 residues of the catalytic domain[3].

Early studies found that protein kinases phosphorylated their target proteins at discrete sites. These enzymes were shown to prefer certain exogenous substrates (such as casein, phosvitin and histones) and only phosphorylated a limited number of available sites. Indeed, Krebs and Fischer showed that phosphorylase kinase only phosphorylated Ser14 in phosphoryl-

ase which contains 64 Ser and Thr residues. Langan's pioneering work on histone phosphorylation demonstrated that several protein kinases may phosphorylate a common substrate at multiple sites. The importance of substrate specificity was further highlighted by the discovery of the cAMP-dependent protein kinase by Walsh and his colleagues. This enzyme did not phosphorylate phosphorylase but did phosphorylate a wide range of exogenous proteins. The idea that the multiple functions of the second messenger cAMP were all mediated by the cAMP-dependent protein kinase implied the enzyme recognized a panel of protein substrates that were subject to hormonal regulation. This raised the question of how protein kinases recognized specific residues out of the numerous hydroxyl groups in their substrates.

It became evident that the local sequence around the phosphorylation site played a vital role in recognition by the cAMP-dependent protein kinase and that arginine residues were involved. The cAMP-dependent protein kinase phosphorylated β casein B at Ser124 in the sequence TERQSLT (nos 120–126) but not in the more common variant β casein A[2] where Ser replaces Arg at position 122 (Ref. 4). This observation suggested that the cAMP-dependent protein kinase substrate recognition motif may be the **RXS*** sequence[†]. Studies in several laboratories in the mid-1970s demonstrated that the cAMP-dependent protein kinase readily phosphorylated short synthetic peptides[5] and provided compelling evidence for the role of Arg residues. Significantly, the synthetic peptide modelled on the liver pyruvate kinase phosphorylation site sequence, LRRASLG (Kemptide), was phosphorylated with kinetic constants comparable to native protein substrates. This data suggests that all of the information necessary for recognition by the cAMP-dependent protein kinase could be present in the local phosphorylation site sequence and that **RRXS** was a preferred motif (reviewed in Ref. 5). While the primary sequence and proximity of Arg residues plays an important role, higher orders of structure can have an overriding influence.

[†]The phosphate acceptor site is indicated **S*** to distinguish it from serine phosphate (S(P)) which may act as a specificity determinant for some protein kinases. Where the specificity determinants are known, less essential residues are marked X and determinant residues are shown in bold. The complete phosphorylation site sequence is given where the major determinants are not known (see Table I).

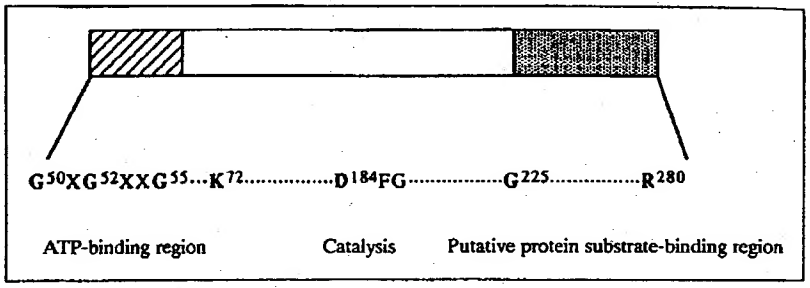

Figure 1
General structure of a protein kinase catalytic domain. Residues conserved in almost all known serine, threonine and tyrosine protein kinases are numbered according to the cAMP-dependent protein kinase catalytic subunit.

For example, lysozyme does not act as a substrate for the cAMP-dependent protein kinase unless it is chemically modified.

Protein kinase specificities

The study of sequences surrounding the local phosphorylation site and the phosphorylation of model peptides has enabled the identification of phosphorylation site motifs for a number of protein kinases (see Table I). Although this is an extensive list, the explosion of protein kinase sequences generated by DNA cloning has outstripped our knowledge of their substrate specificities and identification of natural substrates.

All of the calmodulin-dependent protein kinases studied to date utilize basic residues as specificity determinants. The specificity of phosphorylase kinase was studied in detail by Graves and his colleagues[6]. In the model palindromic peptide LSYRRYSL (nos 1–8), Ser2 is phosphorylated by phosphorylase kinase, whereas the cAMP-dependent protein kinase phosphorylates Ser7 on the carboxyl side of the adjacent arginines[6]. While there is a strong influence of a carboxyl basic residue **S*XR** in the phosphorylase peptide, this is not an absolute requirement since the enzyme can phosphorylate sites without a basic residue in this position. The non-charged adjacent residues may also influence the phosphorylation of model peptides. The myosin light-chain kinases from both smooth and skeletal muscle also show restricted substrate specificity for myosin light chains. While the skeletal muscle isoenzyme will phosphorylate light chains from skeletal, cardiac and smooth muscle, Stull and his colleagues have shown that the smooth muscle enzyme has a strong preference for light chains from the same muscle. The smooth muscle myosin light-chain kinase requires the sequence **KKRXXRXXS***, with the number and spatial arrangement of the basic residues essential for favourable kinetics of phosphorylation and for directing the phosphate to the correct site[7]. In skeletal muscle myosin light chains the local phosphorylation site sequence contains Glu at residue 10 in the sequence PKKAKRRAAEGSS*NVFS (nos 1–17). Synthetic peptide analogs of the native sequence are phosphorylated with low V_{max} values, whereas those containing Arg at position 10, analogous to the smooth muscle light chains, are readily phosphorylated by skeletal muscle enzyme. This is a good example of a negative determinant that is apparent from peptide studies but presumably not accessible in the intact protein. To some extent studying the recognition requirements of protein kinases with highly restricted specificity ranges, such as phosphorylase kinase and the myosin light-chain kinases, is made difficult because of the lack of multiple natural phosphorylation site sequence for comparisons. This is also a problem for the tyrosine kinases. On the other hand, the multifunctional calmodulin-dependent protein kinases have broad specificities and recognize the motif **RXXS*X** in both proteins and peptide substrates[8]. The specificity requirements of the other members of the calmodulin-dependent protein kinase family are being explored in several laboratories.

Protein kinase C has been the subject of numerous substrate specificity studies. The initial studies were carried out on brain enzyme which consists of multiple isoenzymes. All protein kinase C preparations have a requirement for basic residues but there can be considerable variation in the juxtaposition and choice of Arg over Lys around the

phosphorylation site. Synthetic peptides containing the motif XRXXS*XRX tend to be the best substrates and indeed this arrangement is present in the pseudosubstrate autoregulatory region of the enzyme (see below). With the availability of individual recombinant isoenzymes of protein kinase C, Parker and his colleagues are exploring the basic residue requirements of the individual isoenzymes.

The specificity and recognition sites for the cyclic nucleotide-dependent protein kinases have been extensively studied (reviewed in Ref. 5). For the cAMP-dependent protein kinase, the most typical motif is RRXS*X but RXS*X and KRXXS*X are also encountered. The phosphorylation site sequence RRS* occurs in both cardiac troponin and hormone-sensitive lipase, but limited synthetic peptide studies indicate that the Arg adjacent to the Ser(*) is less important than the second Arg. There is also evidence from synthetic peptide studies that more distal Arg residues on the amino-terminal side may have a positive influence. Indeed the heat-stable inhibitor (Walsh inhibitor) of this enzyme has the pseudosubstrate motif GRTGRRNA*I with Ala occupying the equivalent of the Ser phosphate acceptor site. In scanning sequences for cAMP-dependent protein kinase sites the search pattern is RXS*, then RRXS* or KRXXS*, and not S*XR as the carboxy-terminal basic residue tends to be deleterious. The only known examples of RXS* motifs where X is not Arg are exogenous substrates phosphorylated *in vitro*. The sites phosphorylated *in vivo* by the cAMP-dependent protein kinase all contain multiple adjacent Arg residues in the arrangements RRXS* or RRS*, with two having the latter motif. A hydrophobic residue is often, but not exclusively, found after the Ser. While the yeast cAMP-dependent protein kinase specificity differs in several respects, the fact that it can be complemented by the mammalian enzyme in yeast suggests that the differences do not override the natural function. Recognition site specificity of the cGMP-dependent protein kinase has been studied in detail by Glass and his colleagues[5]. From model peptide studies, there is evidence for a requirement for an Arg located on the carboxy-terminal side of the phosphorylated residue in the sequence S*R or T*R; however this is not an absolute requirement as the enzyme has been shown to phosphorylate sites without this motif. The precise requirements are insufficiently clear to allow cGMP-dependent protein kinase phosphorylation sites to be confidently identified by scanning amino acid sequences alone.

As early as 1970 Ribadeau-Dumas *et al.*[9] made correct predictions about the specificity requirements of the casein kinase from the mammary gland Golgi apparatus (S*XE or S*XS(P)) based on the sequence of the polyphosphorylated region in casein. Casein kinase II specificity has been studied extensively, in particular in the laboratories of Pinna and Krebs. This enzyme has a very widespread distribution and recognizes the motifs S*XXE and S*XXS(P). Not every site conforming to the motif is phosphorylated, casein kinase II phosphorylates Ser17 and not Ser18 in the peptide from β casein A², ESLSSSEE (nos 14–21). On the other hand, the mammary gland enzyme phosphorylates Ser18 and not Ser17 in this sequence. Casein kinase I and II have been shown to participate in hierarchal phosphorylation reactions by several groups. Roach and his colleagues found that prior phosphorylation of glycogen synthase by cAMP-dependent protein kinase at Ser7 caused casein kinase I to phosphorylate Ser10, whereas phosphorylation of glycogen synthase by casein kinase II provides the recognition

Table I. Protein kinase phosphorylation site motifs

Protein kinase	Recognition motif	Refs
Serine and threonine kinases		
Phosphorylase kinase	KRKQIS*VR	Chan[6]
Myosin light chain kinase (smooth muscle) Myosin light chain kinase (skeletal muscle)	XKKRXXRXXS*X	Kemp[5]
Myosin-I heavy chain kinase	KXXS*X or RXT*X	Brzeska[10]
Calmodulin-dependent protein kinase I	NYLRRLS*DSNF	Czernik[11]
Multifunctional calmodulin-dependent protein kinase II	XRXXS*X	Pearson[8]
Calmodulin-dependent protein kinase III	RAGET*RFT*DT*RK	Nairn[12]
cAMP-dependent protein kinase (mammalian) (yeast)	XRRXS*X XRRXS*X	Zetterqvist[5]
cGMP-dependent protein kinase	XS*RX	Glass[5]
Protein kinase C (α, β, γ)	XRXXS*XRX	Graff[13]
S6 kinase II	XRXXS*X	Erikson[14]
dsRNA-dependent kinase pp68	SELS*RR	Colthurst[15]
dsDNA-dependent kinase	PEET*QT*QDQPMEEEE	Lees-Miller[16]
Protease activated kinase I & II	AKRRRLSS*LRA	Wettenhall[9]
Cell cycle kinase cdc-28, MPF	XKS*PX or XKT*PX	Langan[18]
Proline-dependent protein kinase	XS*PX or XT*PX	Vulliet[19]
Growth factor regulated kinase	PLT*PSGEA	Countaway[20]
Casein kinase I	XS(P)XXS*X or XEXXS*X	Pinna[5]
Casein kinase II	XS*XXEX	Pinna[5]
Mammary gland casein kinase	XS*XEX or XS*XS(P)X	Pinna[5]
Glycogen synthase kinase-3	XS*XXXS(P)X	Fiol[21]
AMP-activated protein kinase (acetyl CoA carboxylase kinase) (HMG-CoA reductase kinase) (hormone sensitive lipase kinase)	MRSSMS*GLHL MIHNRS*KINL MRRSVS*EAAL	Hardie[22]

Table I. Protein kinase phosphorylation site motifs (continued)

Protein kinase	Recognition motif	Refs
Pyruvate dehydrogenase kinase	S*MSDPGVS*YRYGMGTS*VE	Edelman[23]
Branched chain α-ketoacid dehydrogenase kinase	GHHS*TSDD and SYRS*VDE	Paxton[24]
Heme regulated eIF-2a kinase	LSELS*RR	Kudlicki[25]
Endogenous eIF-4E kinase	KNDKS*KTWQ	Rychlik[26]
Histone H4 kinase I	VKRIS*GLG	Masaracchia[5]
Histone H4 kinase II	AcS*GRGKGG	Masaracchia[5]
Isocitrate dehydrogenase\ kinase (*E. coli*)	GIRS*LNVALR	Thorsness[27]
β-Adrenergic receptor kinase	GYS*S*NGNT*GEQS*G(X)$_{16}$G T*ED(X)$_5$GT*VPS*DNIDS*Q(X)$_3$ S*T*NDS*LL	Hausdorff[28]
Rhodopsin kinase	DEAS*T*T*VKTETS*QVA	Palczewski[29]
Tropomyosin kinase	DNALNDITS*L-COOH	Watson[30]
Tyrosine kinases		
p60$^{v\text{-}src}$	RLIEDNEY*TARQGAK	Geahlen[5]
p56^{lck}	RLIEDNEY*TAREGAK	Geahlen[5]
p40^{thymus}	PEEDGERY*DEDEE	Geahlen[5]
p85$^{gag\text{-}fes}$	REEADGVY*AASGGLR	Geahlen[5]
p90$^{gag\text{-}yes}$	RKIEDNEY*TAREGAK	Geahlen[5]
p120$^{gag\text{-}abl}$	EEKEY*HAE	Geahlen[5]
EGF receptor	TAENAEY*LRVAP	Geahlen[5]
Insulin receptor	TRDIY*ETDY*Y*RK	Geahlen[5]
p75^{liver}	DRVY*VHPF	Geahlen[5]
Spleen tyrosine kinase	EDAEY*AARRRG	Geahlen[5]

[a] R. E. H. Wettenhall and N. Morrice, unpublished.

Ser(P) for glycogen synthase kinase-3. This enzyme recognizes the motif XS*XXXS(P)X. It is of interest that all four of the Ser/Thr kinases studied that utilize Glu or Asp as specificity determinants also recognize Ser(P). There are also examples where phosphorylation at one site by a particular protein kinase can suppress the phosphorylation of a nearby residue by another protein kinase. The nearby phosphorylation sites in the hormone sensitive lipase, MRRSVSEA (nos 560–567) exhibit this behaviour with mutually exclusive phosphorylation[17] by the cAMP-dependent protein kinase (Ser563) and calmodulin-dependent protein kinase II (Ser565). It seems likely that examples of hierarchal phosphorylation will become more frequent as the specificities of additional protein kinase are studied. So far hierarchal phosphorylation has been observed between nearby phosphorylation site sequences, as well as over distances of 25 residues in the case of the regulatory subunit R_{II} phosphorylation by casein kinase II and glycogen synthase kinase-3; it is possible that even more distant interactions involving higher orders of structure may occur.

The recognition sites for a number of tyrosine protein kinases have been examined. Inspection of the known phosphorylation sites indicates that acidic residues are often located near the tyrosine phosphate acceptor site and their importance has been demonstrated with model peptides in several instances. In general, few natural substrates (excluding autophosphorylation) have been reported for tyrosine protein kinases and this has meant that we have not had the benefit of comparisons. In summarizing our knowledge of tyrosine phosphorylation site motifs, Geahlen and Harrison[5] noted: 'Observations drawn from the study of synthetic peptides have frustrated efforts to clearly define primary structural determinants that are involved in the recognition of substrates by tyrosine kinases. It is perhaps some consolation to investigators of tyrosine kinase substrate specificity that not all peptides containing tyrosine residues are substrates'. Clearly some fresh approaches are required.

Phosphorylation site motifs

The progress in recognizing specific phosphorylation site motifs for many protein kinases has led to the expectation of being able to scan protein sequences and identify phosphorylation sites for given protein kinases. However, considerable caution is required as there are too many exceptions at present to accept the phosphorylation site motifs listed in Table I as 'canons' of recognition. We do not know precisely which nominal specificity determinants actually have corresponding residues in protein kinase active sites and which just favour an optimum conformation. Hopefully this dilemma will be short lived with the imminent solution of the X-ray structure of the cAMP-dependent protein kinase complex with substrate and inhibitor peptides. Phosphorylation site sequence studies such as those made by Cohen and his colleagues have been of enormous value in contributing to our knowledge of protein kinase specificity and the development of the concepts of recognition motifs, but their most enduring value is in answering the difficult question of which sites are actually functionally significant *in vivo*. Structures resembling protein kinase phosphorylation site motifs also play an important role in the regulation of protein kinases. These structures, called pseudosubstrate prototopes, are located in the regulatory domains of protein kinases and are responsible for maintaining protein kinases in inactive

forms[3]. The pseudosubstrate sequences typically contain an alanine in place of the serine or threonine found in the phosphorylation site motifs. For protein kinase C, the pseudosubstrate sequence occurs between residues 19 and 31, RFARKGA*LRQKNV, which resembles the substrate motif RXXS*XR (Table I) with Ala25 in place of the phosphate acceptor site. In this case, activation of protein kinase C by binding of diacylglycerol is thought to induce a conformational change that removes the pseudosubstrate structure from the active site allowing access to substrates. One cannot fail to be impressed with the way nature has utilized the same features responsible for substrate recognition to regulate these important enzymes.

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Because of the limit on the number of references it has not been possible to cite many significant contributions, but these can be traced from those given.

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This article is the second in our series on 'conserved protein sequence motifs involved in recognition and targeting'. The series commenced in August 1990.